

ADH4

PDB:3COS

Revision

Revision Type:created

Revised by:created

Revision Date:created

Entry Clone Accession:IMAGE:4722568

Entry Clone Source:MGC

SGC Clone Accession:

Tag:N-terminal His-tag with TEV protease cleavage site

Host:BL21(DE3)-R3-pRARE2

Construct

Prelude:

Sequence:

mhhhhhhssgvdlgtenlyfqsmgTKGKVIKCKAAIAWEAGKPLCIEEVEVAPPKAHEVRIQIIATSLCHTDATVIDSKFEGLAFPV
IVGHEAAGIVESIGPGVTNVKPGDKVIPLYAPLCRKCKFCLSPLTNLGKISNLKSPASDQQLMEDKTSRFTCKGKPVYHFFGTSTF
SQYTVVSDINLAKIDDDANLERVCLLGCGFSTGYGAANNKAVTPGSTCAVFGLGGVGLSAVMGCKAAGASRIIGIDINSEKFKAK
ALGATDCLNPRDLHKPIQEVIIELTKGGVDFALDCAGGSETMKAALDCTTAGWGSCTFIGVAAGSKGLTVFPEELIIGRTINGTFFG
GWKSVDSIPKLVTDYKNKKFNLDALVTHLTPFDKISEAFDLMNQGKSIRTIILIFTag sequence in lowercase

Vector: pNIC28-Bsa4

Growth

Medium:

Antibiotics:

Procedure: 10 µl of a glycerol stock was inoculated into 5ml of TB medium (supplemented with 50 µg/ml Kanamycin) and cultured at 37°C o/n in a shaking incubator (275 rpm). Next day 0.75 ml of o/n culture was used to inoculate 1 litre of TB medium (4x) and grown at 37°C with vigorous shaking (160 rpm) until the culture reaches an OD600 of 2.0. Temperature was reduced to 18°C, and cells were induced with IPTG at a concentration of 0.5 mM, and further cultivated for 16 hrs. Cells were harvested by centrifugation at 6500 rpm for 10 min, and the cell pellet was stored at -20°C until further use.

Purification

Procedure

Column 1: Ni-Sepharose 6 Fast Flow Buffers: Lysis buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 5 mM Imidazole; Wash buffer: 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 5% Glycerol, 30 mM Imidazole; Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole. Note: All the buffers contain 0.5mM TCEP. Procedure: The column was

packed with 2 ml of Ni-Sepharose 6 Fast Flow slurry and equilibrated with 15 ml of binding buffer. The supernatant was loaded onto the column and the column was washed with 20 ml of binding buffer and then 20 ml of washing buffer. The protein was eluted with 10 ml of elution buffer. Column 2: SuperDex 200 16/60 HiLoad (GE/Amersham) Buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5 % glycerol, 0.5 mM TCEP. Procedure: The eluted protein from the Ni-affinity column was loaded on the gel filtration column in GF buffer at 1.0 ml/min on an AKTA Purifier system. Eluted proteins were collected in 1 ml fractions. Enzymatic treatment: TEV cleaved. Column 3: Ni-NTA (TEV clean up) Buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP Procedure: Total 10 mgs of protein was cleaved with 600 µg of TEV protease at 4°C for 48 hours. TEV clean up: The TEV cleaved protein was applied to a 1 ml Ni-NTA column, already equilibrated with gel filtration buffer (10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP). The flow through from the column was collected. The eluate from the column was monitored by SDS gel analysis.

Extraction

Procedure

Lysis buffer: 500 mM NaCl, 5% Glycerol, 50 mM HEPES pH 7.5, 5 mM Imidazole, Complete® protease inhibitors (Roche, 1 tbl/50 ml). Frozen cell pellets were thawed and resuspended in a total volume of 30-40 ml of lysis buffer, and disrupted by using Avestin C-5 microfluidizer, and a supernatant containing the target protein was obtained by centrifugation at 21,000 (rpm) for 45 minutes.

Concentration: The ADH4A protein (in buffer; 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP) was concentrated to 16.19 mg/ml using Vivaspin 10K concentrators and stored at -80°C.

Ligand

NADMassSpec: Corresponds to theoretical mass, as determined by ESI-TOF MS.

Crystallization: Crystals were grown by vapour diffusion in sitting drops at 4°C. Before setting up the experiment NADH was added to the protein to a final concentration of 5mM. A sitting drop consisting of 50 nl protein and 100 nl well solution was equilibrated against well solution containing 0.20M ammonium acetate, 0.1M bis-tris pH 6.5, 25% w/v polyethylene glycol 3350. The crystals were mounted directly from the drop using 20% ethylene glycol as a cryoprotectant and flash-cooled in liquid nitrogen.

NMR Spectroscopy:

Data Collection: Resolution: 2.1 Å, X-ray source: Synchrotron SLS-X10SA, single wavelength.

Data Processing: